Mutation Research, 276 (1992) 3–9

MUTREV 00131

Design and implementation of a collaborative study of the mutagenicity of complex mixtures in *Salmonella typhimurium* *

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Keywords: *Salmonella typhimurium*; Complex mixtures; Bacterial mutagenicity; Ames test; Environmental mixtures, Collaborative study; Interlaboratory variability

Summary

In 1987, the International Programme on Chemical Safety (IPCS) in collaboration with the U.S. Environmental Protection Agency (U.S. EPA) and the U.S. National Institute of Standards and Technology (U.S. NIST) initiated an international collaborative study of the mutagenicity of complex environmental mixtures in the Ames *Salmonella typhimurium* mutation assay. The objectives of this study were: (1) to estimate the inter- and intra-laboratory variability associated with the extraction of mixtures for bioassay, (2) to estimate the inter- and intra-laboratory variability associated with the *Salmonella typhimurium* bioassay when applied to complex mixtures, and (3) to determine whether standard reference complex mixtures would be useful in mutagenicity studies and to evaluate whether reference or certified mutagenicity values determined from this collaborative study should be reported. The complex mixtures used in this study were selected from standard reference materials (SRMs) which had previously been issued by the U.S. NIST as SRM 1597 (coal tar), SRM 1649 (diesel particulate matter)

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* The study objectives, design and implementation plan were developed by members of several ad hoc Steering Groups who met by invitation of the IPCS in Geneva, 1–5 June, 1987; Research Triangle Park, U.S.A., 1–4 December, 1987; and Ottawa, Canada, 1–4 May, 1990.

and SRM 1650 (urban air particulate matter) with certified values for polycyclic aromatic hydrocarbons. These SRM complex mixtures are available to scientists as reference standards for analytical chemistry research and are under consideration as SRMs for mutagenicity studies of complex environmental mixtures. This paper briefly describes the final study design, protocol, selection of the complex mixtures, and implementation of this international study.

Short-term tests are being used in many countries to evaluate the exposure of humans to complex mixtures of mutagens and potential carcinogens from the air, water, soil, and emission sources. These data are often used to make inferences concerning potential risk from exposure to genotoxic mixtures by comparing the mutagenicity of environmental samples. For such a comparison to be valid, it is important to understand the sources of variability associated with determining the mutagenicity of complex mixtures and to minimize such variability.

The Salmonella mutagenicity assay developed by Ames and coworkers (Ames et al., 1975; Maron and Ames, 1983) has been the most frequently used mutagenesis bioassay for studies of complex mixtures. When significant differences are reported in the concentration of mutagens in air, water or emission sources, it is important to determine if these differences are due to: (1) the use of different procedures for sample preparation (such as the extraction of air particles), (2) differences in the protocol used to implement the Salmonella assay system, or (3) differences in the concentration of mutagenic compounds in the environmental samples. The problem of standardizing bioassay techniques is not unique to complex mixtures. In addition to the problems already recognized for conducting such assays with pure compounds, however, evaluation of the mutagenicity of complex mixtures usually requires consideration of both sample collection and preparation procedures. For example, particulate samples often require solvent extraction and water samples may require concentration. After these sample preparation procedures, an additional solvent exchange procedure is often required to transfer the sample to a solvent compatible with the bioassay protocol. These steps can introduce additional variability in the mutagenicity data that is not encountered in the testing of pure chemicals.

The first international collaborative program for the evaluation of short-term tests for carcinogens (de Serres and Ashby, 1981) used a set of test chemicals known to be either carcinogenic or not carcinogenic in rodents to confirm the value of the Salmonella mutation assay as a primary test for potential carcinogens and mutagens. This study demonstrated that some rodent carcinogens were not detected by the Salmonella mutation assay. The results of this study stimulated the International Programme on Chemical Safety (IPCS) to initiate in 1981 the planning of a two-part international collaborative study of short-term tests for carcinogens using a smaller selected set of test chemicals with known carcinogenicity in rodents in a series of in vitro (Part I) and in vivo (Part II) assays. The objective of the in vitro (Part I) collaborative study was to evaluate potential short-term tests for their complementary ability to identify chemical carcinogens that were not readily detected using bacterial assays. 10 chemicals were selected for this study based on their carcinogenicity in rodents and their lack of mutagenicity in the Salmonella mutation assay (Ashby et al., 1985). The primary objective of the in vivo portion of the collaborative study was to determine the ability of a wide range of short-term in vivo assays to discriminate between the carcinogen and noncarcinogen in two structurally related carcinogen/noncarcinogen pairs of chemicals (benzo[*a*]pyrene/pyrene and 2-acetylaminofluorene/4-acetylaminofluorene) (Ashby et al., 1988). Although statistical analyses of the sources of variability in the Salmonella mutation assay have been conducted using data from these and other studies (Margolin et al., 1984), previous international collaborative studies have not provided the participants

with specific protocol guidelines for performing the assays, nor have they specified the doses of the chemicals to be used in the bioassay.

This report describes the design and implementation of an international collaborative study on the mutagenicity of complex mixtures. Other papers in this volume report on the standard reference materials used (May et al., 1992), the results of this collaborative study (Claxton et al., 1992), the statistical analysis and evaluation of the test data (Krewski et al., 1992) as well as the conclusions and recommendations from the final meeting of the investigators and ad hoc Steering Groups (Claxton et al., 1992).

Collaborative study objectives

This collaborative study was undertaken to estimate the mutagenic potency of complex mixtures under conditions where each laboratory used similar, well defined protocols. The primary objectives of this collaborative trial were: (1) to estimate the intra- and inter-laboratory variability in extraction procedure(s) used for preparing test samples of complex mixtures for chemical and mutational studies; (2) to determine the intra- and inter-laboratory variation in mutagenic potency values obtained for standard complex environmental mixtures; and (3) to determine if one or more complex mixtures available from the U.S. National Institute of Science and Technology (NIST) as Standard Reference Materials (SRMs) can be used as a reference material in bioassay studies of complex environmental mixtures.

Secondary objectives for this collaborative study were: (1) to establish 'marker compounds' for reference complex mixtures that can be used to determine extraction efficiencies and to monitor for stability; (2) to estimate the inter- and intra-laboratory variation in mutagenic potency of reference compounds used as positive controls for these studies; and (3) to establish reference or 'certified' bioassay data for issuance with SRMs for biological studies for a comparative reference standard.

Standard reference materials (SRMs)

The SRMs available through the U.S. National Institute for Standards and Technology (formerly the U.S. National Bureau of Standards) which were considered for this study were those complex environmental mixtures which had certified values for certain organics such as PAHs. These SRMs included: (1) a marine river sediment sample; (2) an urban air particle sample (SRM 1649, urban dust/organics); (3) a diesel particle sample (SRM 1650, diesel particulate matter); and (4) a coal tar sample (SRM 1597, complex mixture of polycyclic aromatic hydrocarbons from coal tar). These complex mixture SRMs were made available through the cooperation of the U.S. Environmental Protection Agency and the U.S. National Institute for Standards and Technology.

Pilot study

A pilot study was conducted to select the final SRMs for the study, the extraction protocol (method and solvents), and the dosages to be used in the Salmonella assay. The pilot study also provided an opportunity to evaluate the shipping procedures. The laboratories participating in the pilot studies were the U.S. EPA, Genetic Bioassay Branch laboratory (Dr. L. Claxton), the National Institute of Public Health laboratory in Tokyo, Japan (Dr. H. Matsushita) and the University of Stockholm in Stockholm, Sweden (Dr. G. Löfroth).

The pilot study results on evaluation of the marine sediment sample led to the conclusion that the weak mutagenic activity of this sample would make it inappropriate for the collaborative study. The final three mixtures selected for the collaborative study were SRM 1649 (air particulate matter), SRM 1650 (diesel particulate matter), and SRM 1597 (coal tar).

Study design

The experimental design for the collaborative study was established at the meeting of the ad hoc Technical Steering committee in December, 1987. One of the primary objectives of the study was to estimate between and within laboratory sources of variability in bioassay results. The final experimental design called for each laboratory to conduct replicate extractions of organic material from samples of urban air and diesel particulate

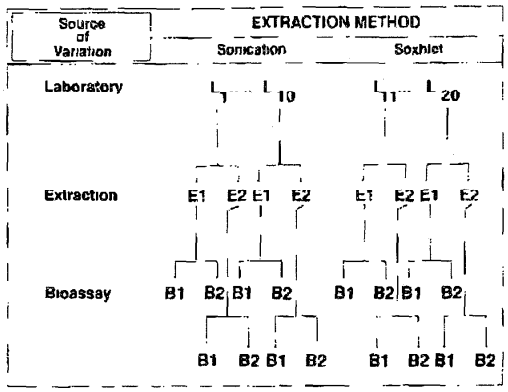

Fig. 1. Overview of the experimental design used for determining sources of variation in the mutagenic potency of urban air particles and diesel particles

matter provided by NIST; each such extract would then be subject to replicate bioassays done on different days (Fig. 1). This would provide 4 estimates (2 extractions × 2 bioassays per extraction) of the mutagenic potency of SRM 1649 and SRM 1650. This was repeated using two strains of Salmonella (TA98 and TA100) with and without metabolic activation using rat liver S9. Two different methods (Soxhlet and sonication) of extracting organics were used; half of the 20 participating laboratories were asked to use sonication, while the remaining 10 used the Soxhlet method.

This design provided for an assessment of between and within laboratory sources of variation in the mutagenic potency of urban air and diesel particulate matter. Variation among the average values of mutagenic potency reported by individual laboratories provided an indication of interlaboratory variation; variation between replicate extractions and replicate bioassays within each laboratory yielded an estimate of these two sources of intralaboratory variation. Separate analyses of the data for laboratories using sonication and Soxhlet extractions facilitated a comparison of the magnitude of inter- and intra-laboratory variation in mutagenic potency using these two extraction methods.

Since the coal-tar samples did not require extraction prior to bioassay, 4 replicate bioassays were conducted within each laboratory on SRM 1597. This was also true of the positive controls benzo[*a*]pyrene (tested without S9 only) and 1-nitropyrene (tested with S9 only).

The Technical Steering Committee chose this design because it represented a minimal design allowing for an assessment of the sources of variation of most interest, and for a direct comparison between the sonication and Soxhlet methods of extracting organics. A more demanding protocol in which each participating laboratory would use both extraction methods was considered, but not adopted because it would double the number of bioassays required and because not all laboratories expressed confidence in their ability to implement both extraction procedures.

The study protocol also called for participating laboratories to report the percentage of organic material extracted from the samples of urban air and diesel particulate matter. This was done using replicate extractions of separate subsamples of SRM 1649 and 1650. This provided for estimation of inter- and intra-laboratory variation in extraction efficiency which, because doses for use in the Salmonella assay were established in proportion of the mass of the unextracted sample, could account for part of the observed variation in mutagenic potency.

Each laboratory returned its test results entered on standard data forms to the central bioassay laboratory for entry into a computerized data base. Bioassay results and estimates of mutagenic potency (as measured by the slope of the initial linear component of the dose–response curve) were then returned to participating laboratories for data validation. During the course of the collaborative trial, the identity of the SRMs and the positive controls was not disclosed to the participating laboratories. By referring to participating laboratories only by their code number (from 1 to 20) in the analysis of the study results, the identity of individual laboratories was not revealed.

Chemistry reference laboratory

The Center for Analytical Chemistry at NIST in Gaithersburg, MD (U.S.A.), under the direction of Dr. W. May served as the chemical reference laboratory for the collaborative study. The NIST laboratory was responsible for sample

preparation, shipment, and chemical analyses on extracts returned from participating laboratories. At a minimum, the chemical analysis included a gravimetric analysis of the extractable mass from the mixtures requiring extraction (SRMs 1650 and 1649). Analysis of the following marker compounds was recommended: (1) pyrene; (2) 1-nitropyrene (1-NP); (3) benzo[*a*]pyrene (BaP); (4) 1,6-pyrenequinone; (5) benzo[*e*]pyrene (BeP); (6) benzo[*ghi*]perylene (BghiP); and (7) 9-fluorenone. The marker compounds were selected to measure extraction efficiency based on polarity; they are not viewed as reflecting the mutagenic potency of the SRMs. The determination of the BaP/BeP ratio or BaP/BghiP ratio was proposed as a simple means of screening extracts for potential degradation. Each participating laboratory was encouraged to perform its own chemical analysis for these 'marker compounds' as an optional part of the study. With the exception of BaP and 1-NP, these reference chemicals were not sent to each participating laboratory.

One shipment of all material needed for this study was made by the chemical reference laboratory in 1989 to each participating laboratory. This shipment of coded samples included: (1) sufficient quantities of the 2 particulate matter SRMs (SRM 1649 and SRM 1650) to allow for two separate replicate extractions, plate incorporation bioassay and optional studies discussed below; (2) the coal tar extract (SRM 1597); and (3) two reference chemicals, benzo[*a*]pyrene (BaP) and 1-nitropyrene (NP) which served as positive controls in the data analysis. Materials which were not provided and shipped to participating laboratories included the *Salmonella typhimurium* tester strains, S9 activation system, and other components of the bioassay protocol including the positive control chemicals normally used in each laboratory. Chemicals such as solvents used in extraction and marker chemicals for chemical analysis were also not provided to laboratories as part of this shipment, although standard reference marker chemicals were provided on request.

Central bioassay laboratory

The Genetic Bioassay Branch's laboratory at the U.S. Environmental Protection Agency (EPA), Research Triangle Park, NC (U.S.A.) served as the central bioassay laboratory under the direction of Dr. L.D. Claxton. The EPA laboratory received all of the returned data sheets and samples. Containers of the returned extracts were in many cases damaged, therefore precluding the planned bioassay and chemical analysis of these samples. The undamaged samples returned for chemical analysis were shipped to NIST's central chemistry laboratory. The returned data sheets were reviewed and entered into a computerized data base for statistical analysis. The computerized data sheets were sent to each laboratory for review and correction. Inquiries were made to any participating laboratories who sent unclear or incomplete information.

Participating laboratories

Participating laboratories were solicited by sending letters of inquiry and application forms to laboratories who had published research on the mutagenicity of complex mixtures using Salmonella typhimurium mutagenesis methods. The final 20 participating laboratories (Claxton et al., 1992) were selected from the respondents based upon their demonstrated capabilities in performing both the chemical extractions and bioassay protocol. The responsibilities of the participating laboratories were to follow the protocol provided with the coded samples, document specified procedures, and report the data and other information requested on forms provided within a specified time period during 1989.

Extraction methods

Solvent extraction is one of the most common preparation methods used to prepare complex environmental samples for bioassay. Often the optimal extraction solvent is not compatible with the bioassay method so an additional solvent exchange step is required before the bioassay. The two particulate samples, SRM 1649 (urban air particulate matter) and SRM 1650 (diesel particulate matter) required solvent extraction. Based on the pilot study results and other published studies of these samples (May et al., 1992), dichloromethane was chosen as the extraction solvent.

Two extraction methods were compared in this study, Soxhlet extraction and sonication. 10 of the laboratories were assigned Soxhlet extraction and the other 10 laboratories were assigned sonication. Each laboratory was directed to use the protocol normally followed in their laboratory for these procedures. The protocol specified that extracted organic matter was to be evaporated nearly to dryness and solvent exchanged into dimethyl sulfoxide (DMSO) for bioassay.

The third complex mixture sample, SRM 1597 (coal tar) was supplied as a liquid sample in toluene. The participating laboratories were instructed to also solvent exchange this sample into DMSO.

Mutagenesis bioassay method

The mutagenesis bioassay specified to each participating laboratory was the *Salmonella typhimurium*/microsome plate incorporation protocol as described by Maron and Ames (1983) and guidelines published by Claxton et al. (1987). Instructions to the participating laboratories specified the use of *Salmonella typhimurium* strains TA98 and TA100 and the use of minimal media plates made with Difco agar. The instructions specified the use of an Aroclor-1254 induced rat-liver homogenate activation system to be prepared as described by Maron and Ames (1983). The doses, strains and activation conditions were specified for each sample as described by Claxton et al. (1992).

Optional research studies

Once the required part of the collaborative study was completed and the data submitted, each laboratory was encouraged to perform optional studies which are reported independently in this volume. Several optional studies of interest to the ad hoc Steering Group were suggested to the participating laboratories as follows: (1) evaluation of the plate-incorporation vs. pre-incubation protocol, (2) more extensive chemical analysis of the extracts under study including fractionation studies, (3) evaluation of other in vitro bioassays, and (4) evaluation of other extraction solvents.

Acknowledgements and disclaimer

The authors acknowledge the support of all of the laboratories and investigators who donated their time to participate in this collaborative study. The research described in this paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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